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## Original Article

# Long-chain bases from sea cucumber mitigate endoplasmic reticulum stress and inflammation in obesity mice

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## ABSTRACT

Endoplasmic reticulum (ER) stress and inflammation can induce hyperglycemia. Long-chain bases (LCBs) from sea cucumber exhibit antihyperglycemic activities. However, their effects on ER stress and inflammation are unknown. We investigated the effects of LCBs on ER stress and inflammatory response in high-fat, fructose diet-induced obesity mice. Reactive oxygen species and free fatty acids were measured. Inflammatory cytokines in serum and their mRNA expressions in epididymal adipose tissues were investigated. Hepatic ER stress-related key genes were detected. c-Jun NH<sub>2</sub>-terminal kinase and nuclear factor  $\kappa$ B inflammatory pathways were also evaluated in the liver. Results showed that LCBs reduced serum and hepatic reactive oxygen species and free fatty acids concentrations. LCBs decreased serum proinflammatory cytokines levels, namely interleukin (IL)-1 $\beta$ , tumor necrosis factor- $\alpha$ , IL-6, macrophage inflammatory protein 1, and c-reactive protein, and increased anti-inflammatory cytokine IL-10 concentration. The mRNA and protein expressions of these cytokines in epididymal adipose tissues were regulated by LCBs as similar to their circulatory contents. LCBs inhibited phosphorylated c-Jun NH<sub>2</sub>-terminal kinase and inhibitor  $\kappa$  kinase  $\beta$ , and nuclear factor  $\kappa$ B nuclear translocation. LCBs also inhibited mRNA expression of ER stress markers glucose regulated protein, activating transcription factor 6, double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase, and X-box binding protein 1, and phosphorylation of eukaryotic initiation factor- $\alpha$  and inositol requiring enzyme 1 $\alpha$ . These results indicate that LCBs can alleviate ER stress and inflammatory response. Nutritional supplementation with LCBs may offer an adjunctive therapy for RE stress-associated inflammation.

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E-mail address: [hushiwei@163.com](mailto:hushiwei@163.com) (S. Hu).<sup>1</sup> The first two authors contributed equally to this work.<http://dx.doi.org/10.1016/j.jfda.2016.10.011>1021-9498/Copyright © 2016, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Low-grade inflammation in adipose tissue is considered an important procedure in the development of obesity-related comorbidities, including diabetes mellitus, hyperglycemia, and insulin resistance [1,2]. The chronic inflammation in adipose tissue is powerfully augmented through the secretion of free fatty acids (FFAs) and deleterious inflammatory cytokines, such as interleukin (IL)-1 $\beta$  and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) [3]. Elevated proinflammatory cytokines and excessive oxidative stress maintain a proinflammatory environment, and firstly cause tissue damage in the liver and lead to further activation of inhibitor  $\kappa$  kinase  $\beta$  (I $\kappa$ K $\beta$ ), c-Jun NH<sub>2</sub>-terminal kinase (JNK), and other serine kinases [4]. These serine kinases, in turn, lead to the production of IL-1 $\beta$ , TNF- $\alpha$ , FFAs, reactive oxygen species (ROS), etc. [5,6]. Recent studies suggested that endoplasmic reticulum (ER) stress was an important contributor to chronic tissue inflammation [7,8]. ER is the organelle for the synthesis, folding and trafficking of secretory and membrane proteins [9]. Disruption of ER homeostasis results in an adaptive unfolded protein response (UPR) triggered by glucose regulated protein (GRP78/Bip) pathway, intended to restore the capacity of ER and alleviate this stress [10]. Three distinct ER transmembrane proteins initiate the canonical UPR: activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE1), and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) [4,11]. Once the UPR is unable to prevent the accumulation of unfolded proteins, ER stress response occurs. JNK and nuclear factor (NF)  $\kappa$ B pathways are directly involved in ER stress-induced inflammatory response. For example, JNK and NF $\kappa$ B can be activated by IRE1 through the pro-inflammatory cytokine, TNF- $\alpha$  [12]. phosphoenolpyruvate kinase/eukaryotic initiation factor  $\alpha$  (eIF2 $\alpha$ )-triggered ER stress directly promotes NF $\kappa$ B nuclear translocation [13], which subsequently increases the productions of TNF- $\alpha$  and IL-6 [14].

Long-chain bases (LCBs), also called sphingoid bases, are the simplest members in the family glycosphingolipids. Current studies showed that LCBs possessed several bioactivities, such as antioxidation, antitumor, improvement in type 2 diabetes, and inhibition of keratinocyte differentiation [15–18]. LCBs from marine organisms exhibit better bioactivities because of their special environment [19]. Therefore, raising contents have been drawn in LCBs extracted from sea cucumbers (SC-LCBs). SC-LCBs were reported to induce apoptosis in human hepatoma HepG2 cells through phosphorylated protein kinase B and death receptor-5 [20]. SC-LCBs also ameliorated glucose tolerance and hepatic triglyceride content in obese mice [21,22]. However, the effects of LCBs on ER stress associated with inflammatory response have not been verified. Sphingoid bases from plants were proved to inhibit TNF- $\alpha$  and IL-8 levels in human endothelial cells, but the mechanism is not understood [23]. Therefore, the present study was conducted to evaluate whether LCBs could influence ER stress and inflammation or not. In addition, the molecular mechanism by which LCBs altered ER stress-induced inflammatory signal transduction was also investigated.

## 2. Methods

### 2.1. Preparation of LCBs

Dried sea cucumber, *Cucumaria frondosa*, was purchased from Nanshan Aquatic Products Market (Qingdao, China). LCBs were extracted and analyzed as the previous reports [24]. Briefly, total lipids were extracted from sea cucumber using chloroform–methanol (2:1 v/v); 4M KOH in methanol was added into the total lipids and performed 2 hours at 37°C. Extraction was subsequently performed with chloroform–methanol–distilled water (2:1:0.9 v/v/v), and the chloroform layer was collected. After vacuum concentration, the lipids were under HCl acidolysis 16 hours at 80°C, and then was extracted using *n*-hexane and subsequently diethyl ether, respectively. The crude LCBs were obtained from diethyl ether. To gain pure LCBs, HPLC was performed using an Agilent 1100 HPLC system (Santa Clara, CA, USA) with diode array detection, and a TSK gel ODS-80Ts. The purity of LCBs was 96.4% using HPLC system with a diode array detector. The yield of LCBs was about 1.47%. Their molecular weights were in the range of 238.4–320.5 analyzed by the electrospray ionization-MS method. The components and the main chemical structure of *C. frondosa* LCBs are shown in Figure 1.

### 2.2. Animal experiments

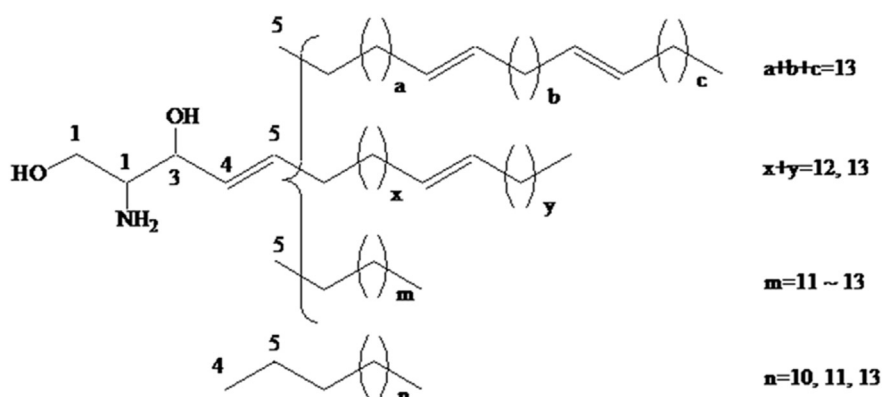
Five-week-old male C57BL/6J mice (licensed ID: SCXK2011-0011) were obtained from Vital River Laboratory Animal Center (Beijing, China). Animals ( $n = 12/\text{group}$ ) were housed in individual cages under a 12-hour light/dark cycle at  $23 \pm 1^\circ\text{C}$  daily. The animals were assigned to four groups: control group (maintained on a control diet for 12 weeks); HFFD group [fed high-fat fructose diet (HFFD) diet for 12 weeks]; and low and high dosage of LCBs groups (fed HFFD diet for 4 weeks, and then continuously fed HFFD diet with LCBs at a diet supplement dosage of 0.008% and 0.025% for 8 weeks, respectively). The control diet consisted of 20% protein, 5% fat, and no fructose, whereas the HFFD consisted of 20% protein, 25% fat, and 20% fructose. All experimental protocols used in this study were approved by animal ethics committee as per the guidelines of the Standards for Laboratory Animals of China (GB 14922-94, GB 14923-94, and GB/T 14925-94).

### 2.3. Fasting serum ROS, FFAs, and inflammatory factors assay

Serum ROS, FFAs, TNF- $\alpha$ , C-reactive protein (CRP), macrophage inflammatory protein 1 (MIP-1), IL-1 $\beta$ , IL-6, and IL-10 levels were assessed with their corresponding enzyme-linked immunosorbent assay kits (Invitrogen, Carlsbad, CA, USA).

### 2.4. Hepatic ROS and FFAs concentrations analysis

Hepatic ROS and FFAs levels were detected as in our previous study [4]. Briefly, the liver was homogenized in hydroxylpiperazine ethane sulfonic acid (HEPES) buffered saline



m/z	Structure	Relative content	m/z	Structure	Relative content
238.4	d16:0	2.73	250.5	t17:0	9.33
262.5	t18:1	8.62	265.5	d17:1	14.11
274.5	d19:3	2.21	278.4	d19:1	3.70
280.5	d18:2	27.95	286.5	d17:1	2.79
292.5	d20:1	8.40	294.5	d19:2	2.27
298.5	d19:0	6.94	306.5	d21:1	3.57
310.5	d20:1	4.88	320.5	d22:1	2.50

Figure 1 – The structures of long-chain bases from the sea cucumber, *Cucumaria frondosa*.

(containing 140mM NaCl, 5mM KCl, 1mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, 10mM HEPES, and 140mM glucose, pH 7.4). The supernatant for ROS and FFA analysis was obtained by centrifugation at  $12,000 \times g$  for 30 minutes. ROS and FFA concentrations were tested using their corresponding enzyme-linked immunosorbent assay kits (Invitrogen, Carlsbad, CA, USA).

## 2.5. Quantitative real-time polymerase chain reaction analysis

RNA in the liver and epididymal adipose tissues was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from 1  $\mu$ g of RNA using a polymerase chain reaction (PCR) thermocycler (iQ5; Bio-Rad, Hercules, CA, USA). PCR was tested by amplification of 15 ng cDNA in a 25- $\mu$ L reaction containing SYBR-Green mix (Invitrogen, Carlsbad, CA, USA) using iQ5 Real-Time PCR System. The mixtures were incubated for a predenaturation at 95°C for 10 minutes, followed by 45 PCR cycles: 15 seconds at 95°C, 20 seconds at 60°C, 30 seconds at 72°C. [Supplementary Table 1](#) shows the primers used in this study. Data were analyzed using the software of iCycler iQ5. The levels of expression of inflammatory cytokines genes and ER stress marker genes were normalized to that of  $\beta$ -actin.

## 2.6. Obtainment of cytosolic and nuclear fractions

For detecting NF $\kappa$ B, cytosolic and nuclear fractions in epididymal adipose tissue were obtained according to our previous study [25]. Briefly, epididymal adipose tissue was homogenized in extraction buffer (250mM NaCl, 20mM NaOH, 50mM

HEPES, 40mM sucrose, and protease inhibitor cocktail) and centrifuged repeatedly to obtain the supernatant, which contained the cytosolic fraction. The other liver was homogenized in buffer A (containing 25mM Tris-HCl, 130mM NaCl, and 5mM KCl, pH 7.4) and centrifuged to gain a pellet. The pellet was lysed in buffer B (containing 10mM KCl, 10mM ethylene diamine tetraacetic acid, 10mM HEPES, 0.1mM ethylene glycol tetraacetic acid, 1mM phenylmethanesulfonyl fluoride (PMSF), and 1mM dithiothreitol, pH 7.9), incubated on ice for 20 minutes, centrifuged to obtain the rough nuclei. The rough nuclei was re-extracted with buffer C (containing 400mM NaCl, 1mM ethylene diamine tetraacetic acid, 20mM HEPES, 1mM ethylene glycol tetraacetic acid, 1mM phenylmethanesulfonyl fluoride, and 1mM dithiothreitol, pH 7.9), incubated on ice for 2 hours, and then centrifuged to obtain the supernatant nuclear fraction.

## 2.7. Western blot analysis

Cellular protein in the liver or epididymal adipose tissue was obtained using IP lysis buffer. The proteins were resolved by 10% polyacrylamide gels containing sodium dodecyl sulfate, transferred to polyvinylidene fluoride membranes, and blotted with 5% bovine serum albumin. Protein was incubated overnight at 4°C with primary antibody (Promega, Madison, WI, USA), and subsequently incubated for 2 hours with horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Danvers, MA, USA). Immunodetection was carried out using electrochemical luminescence kit and then normalized with  $\beta$ -actin or the total corresponding protein for the phosphorylation, and proliferating cell nuclear antigen for nuclear NF $\kappa$ B protein.

## 2.8. Statistical analysis

For all experiments, statistical analysis using SPSS version 17.0 software was performed by a one-way ANOVA followed by Tukey's test. Differences were considered statistically significant at  $p < 0.05$ .

## 3. Results

### 3.1. LCBs reduce body weight in obesity mice

HFFD caused a significant increase in body weight gain in C57BL/6J mice. Although a 16.1% reduction in body weight gain was exhibited in low dosage of LCBs group (Table 1), this was not statistically significant compared with HFFD-fed mice ( $p > 0.05$ ). High dosage of LCBs significantly decreased body weight gain by 35.0% in HFFD-fed mice. Meanwhile, epididymal adipose weight was lowered by 48.0% in the high dose of LCBs group compared with the HFFD group.

### 3.2. LCBs decrease ROS and FFAs concentrations

Table 1 shows the effects of LCBs on ROS and FFAs concentrations in serum and liver. In HFFD group, mice showed significantly higher levels of ROS and FFAs concentrations compared to control group. High dosage of LCBs addition to HFFD-fed mice significantly reduced ROS and FFAs concentrations by 26.7% and 28.2% in serum and by 37.9% and 46.7% in the liver, respectively. Moreover, treatment with high dosage of LCBs also lowered serum FFAs concentration ( $p < 0.05$ ).

### 3.3. LCBs inhibit inflammatory response

TNF- $\alpha$ , CRP, MIP-1, IL-1 $\beta$ , and IL-6 are the main proinflammatory cytokines, and IL-10 is an anti-inflammatory factor. As shown in Table 1, high dosage of LCBs caused an increase by 31.0% in serum IL-10 and reductions in serum

TNF- $\alpha$ , CRP, MIP-1, IL-1 $\beta$ , and IL-6 by 43.3%, 25.5%, 35.0%, 13.4%, and 21.7%, respectively. Low dosage of LCBs also significantly increased serum IL-10 level and reduced TNF- $\alpha$  concentration ( $p < 0.05$ ). These results suggest that LCBs can alleviate inflammatory response.

### 3.4. LCBs regulate inflammatory cytokines expressions

We next detected the mRNA and protein expressions of inflammatory cytokines in epididymal adipose tissue of LCBs-treated mice. Figure 2A showed 68.5% increase in IL-10 mRNA expression in high dosage of LCBs-treated mice compared with HFFD animals. TNF- $\alpha$ , CRP, MIP-1, IL-1 $\beta$ , and IL-6 gene mRNA expressions were significantly decreased in high dosage of LCBs-treated mice ( $p < 0.05$ ,  $p < 0.01$ ). Meanwhile, as shown in Figure 2B and C, high dosage of LCBs significantly lowered the protein expressions of TNF- $\alpha$  and IL-1 $\beta$  ( $p < 0.05$ ,  $p < 0.01$ ). Both low and high dosage of LCBs promoted IL-10 protein expression in epididymal adipose tissue of obesity mice ( $p < 0.05$ ,  $p < 0.01$ ). These indicate that LCBs alleviate inflammation via normalization of inflammatory cytokine productions.

### 3.5. LCBs activate JNK and NF $\kappa$ B pathways

Inflammatory signal is transferred through a series of inflammation-related signal cascades, in which JNK and I $\kappa$ B/NF $\kappa$ B pathways are the main signals. Aforementioned data suggest that LCBs could improve inflammatory response. Therefore, we subsequently tested the effects of LCBs on JNK and I $\kappa$ B/NF $\kappa$ B inflammatory pathways in epididymal adipose tissue. As shown in Figure 3A, high dosage of LCBs caused a significant reduction in JNK1 phosphorylation ( $p < 0.01$ ). High dosage of LCBs also lowered the p-I $\kappa$ B protein abundance ( $p < 0.01$ ; Figure 3B). Figure 3C and D show the nuclear and cytoplasmic protein expression of NF $\kappa$ B in epididymal adipose tissue of obesity mice. Higher NF $\kappa$ B expression in the cytoplasmic fraction and lower in the nuclear fraction were exhibited in the LCBs supplementary groups than in the

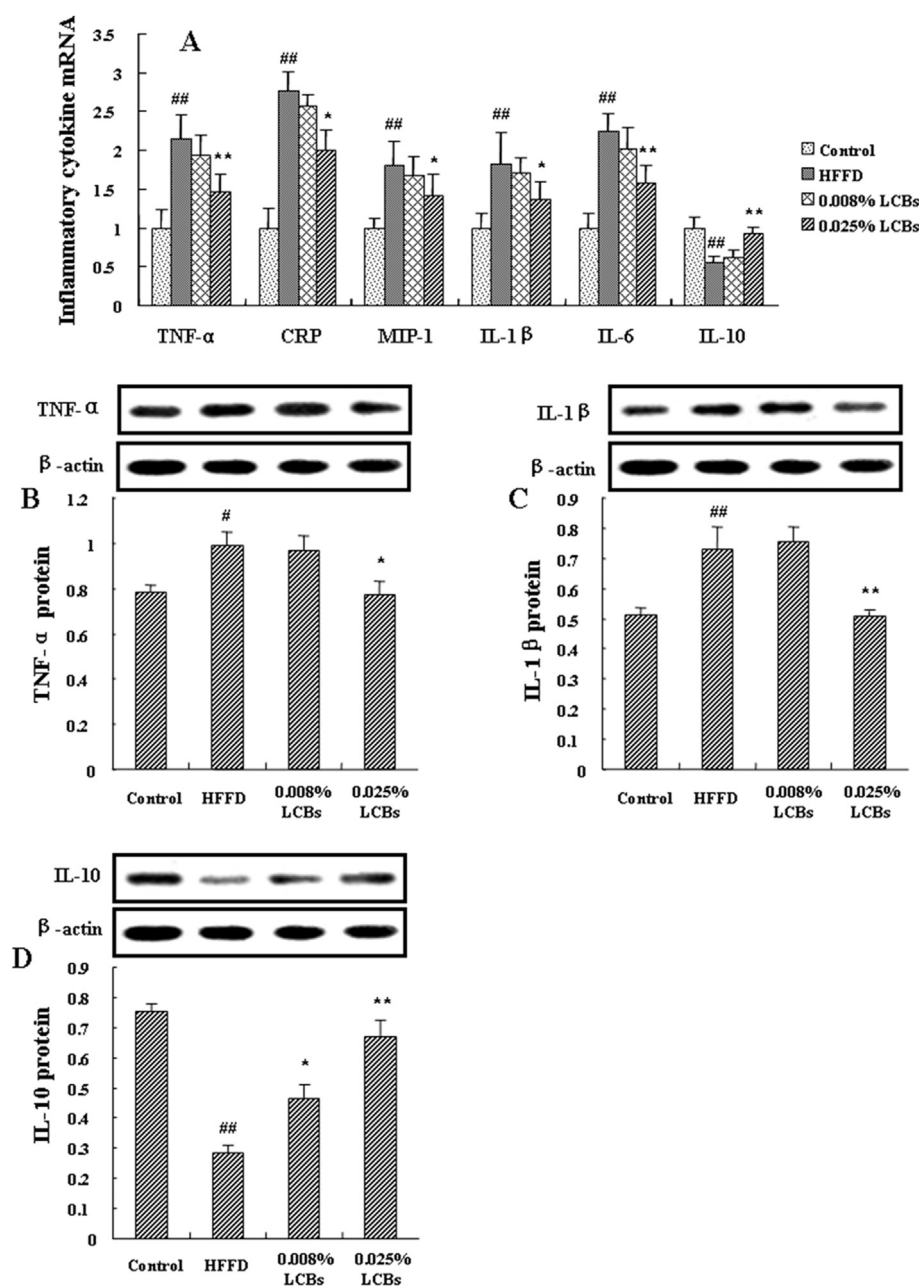
**Table 1 – Effects of LCBs on body weight, serum inflammatory cytokines, and serum and hepatic ROS and FFAs concentrations in obese mice.**

	Control	HFFD	0.008% LCBs	0.025% LCBs
Body weight gain (g)	15.5 $\pm$ 1.7	28.6 $\pm$ 2.1 <sup>##</sup>	24.0 $\pm$ 2.3	18.6 $\pm$ 1.1 <sup>**</sup>
Epididymal adipose weight (g)	0.554 $\pm$ 0.083	1.67 $\pm$ 0.20 <sup>##</sup>	1.35 $\pm$ 0.22	0.868 $\pm$ 0.127 <sup>**</sup>
Serum TNF- $\alpha$ (pg/mL)	287 $\pm$ 13	536 $\pm$ 30 <sup>##</sup>	479 $\pm$ 25 <sup>*</sup>	304 $\pm$ 19 <sup>**</sup>
Serum CRP (ng/mL)	92.3 $\pm$ 6.6	137 $\pm$ 18 <sup>##</sup>	124 $\pm$ 10	102 $\pm$ 8 <sup>*</sup>
Serum MIP-1 (pg/mL)	28.9 $\pm$ 1.4	56.3 $\pm$ 3.4 <sup>##</sup>	50.4 $\pm$ 2.1	36.6 $\pm$ 2.4 <sup>**</sup>
Serum IL-1 $\beta$ (pg/mL)	54.3 $\pm$ 6.1	71.8 $\pm$ 4.4 <sup>##</sup>	67.5 $\pm$ 3.7	62.2 $\pm$ 4.1 <sup>*</sup>
Serum IL-6 (pg/mL)	32.9 $\pm$ 3.7	52.5 $\pm$ 4.4 <sup>##</sup>	47.7 $\pm$ 3.6	41.1 $\pm$ 2.4 <sup>**</sup>
Serum IL-10 (pg/mL)	122 $\pm$ 7	70.1 $\pm$ 5.7 <sup>##</sup>	102 $\pm$ 7 <sup>*</sup>	91.8 $\pm$ 8.5 <sup>**</sup>
Serum ROS (U/mL)	164 $\pm$ 8	258 $\pm$ 13 <sup>##</sup>	233 $\pm$ 11	189 $\pm$ 6 <sup>**</sup>
Serum FFAs ( $\mu$ mol/L)	236 $\pm$ 14	376 $\pm$ 20 <sup>##</sup>	315 $\pm$ 12 <sup>*</sup>	270 $\pm$ 10 <sup>**</sup>
Hepatic ROS (U/mg)	48.0 $\pm$ 3.1	97.2 $\pm$ 6.7 <sup>##</sup>	83.8 $\pm$ 6.2	60.4 $\pm$ 5.3 <sup>**</sup>
Hepatic FFAs ( $\mu$ mol/mg)	0.449 $\pm$ 0.016	1.38 $\pm$ 0.09 <sup>##</sup>	1.16 $\pm$ 0.11	0.735 $\pm$ 0.039 <sup>**</sup>

The obesity model mice were established by fed HFFD. Data are presented as mean  $\pm$  standard deviation ( $n = 12$ /group). Multiple comparisons were done using one way ANOVA. <sup>##</sup>  $p < 0.01$  versus control; <sup>\*</sup>  $p < 0.05$ , <sup>\*\*</sup>  $p < 0.01$  versus HFFD.

CRP = C-reactive protein; FFA = free fatty acid; HFFD = high-fat fructose diet; IL = interleukin; LCBs = long-chain bases; MIP = macrophage inflammatory protein; ROS = reactive oxygen species; TNF = tumor necrosis factor- $\alpha$ .





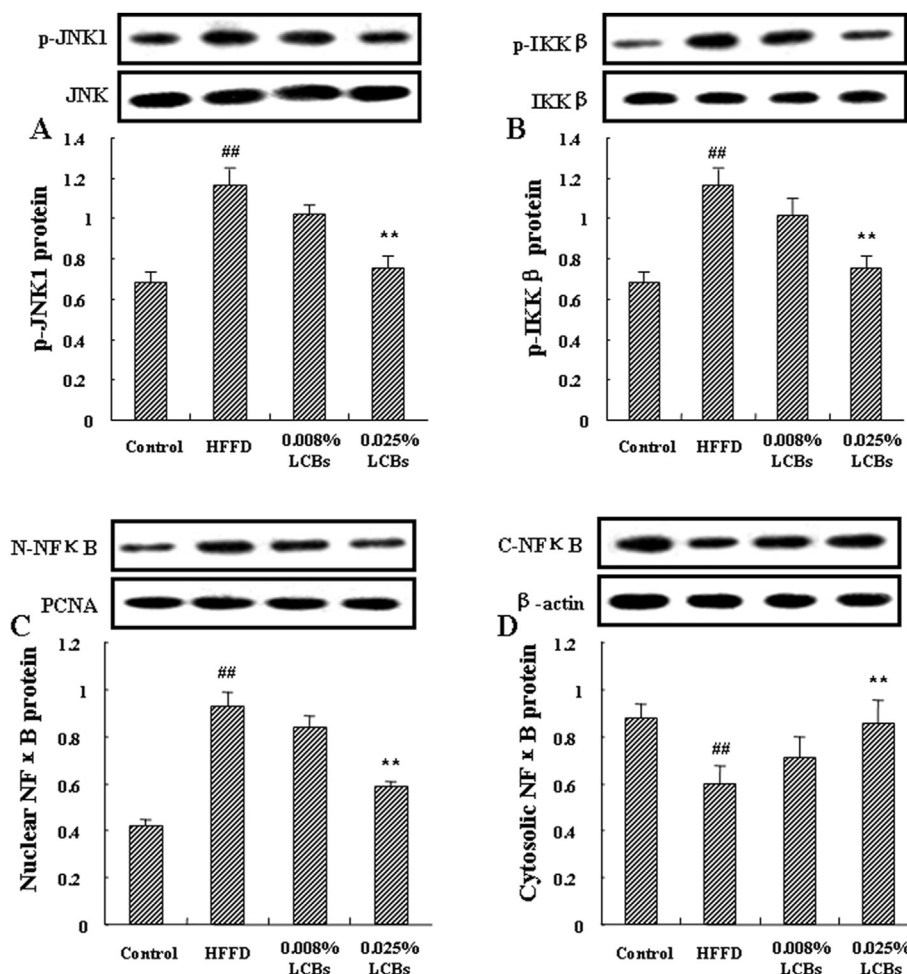
**Figure 2 – Effects of long-chain bases on inflammatory cytokines mRNA and protein expression in epididymal adipose tissue of obesity mice. (A) inflammatory cytokines mRNA; (B) tumor necrosis factor- $\alpha$  protein; (C) interleukin-1 $\beta$  protein; (D) interleukin-10 protein. Data are expressed as mean  $\pm$  standard deviation ( $n = 12$ /group). Multiple comparisons were done using one-way ANOVA analysis followed by Tukey test. <sup>##</sup>  $p < 0.01$  versus control; <sup>\*</sup>  $p < 0.05$ , <sup>\*\*</sup>  $p < 0.01$  versus high-fat fructose diet (HFFD).**

obesity mice, suggesting that LCBs induced NF $\kappa$ B translocation and activation. These results indicate that inactivation of JNK and I $\kappa$ B/NF $\kappa$ B pathways by LCBs may be the signal response of improvement of inflammatory response.

### 3.6. LCBs improve ER stress

Prolonged or unmitigated activity of UPR can induce inflammation. As the inflammatory signaling was inhibited by LCBs in HFFD-fed mice, we next determined LCBs-influenced ER

stress. The initiated gene for UPR, *Bip*, can cope with the interrupted ER homeostasis through a series of stressors. As shown in Figure 4A, increased *Bip* mRNA expression was observed in obesity mice, and the elevation was significantly reduced in the liver obtained from LCBs-treated mice ( $p < 0.01$ ). Three arms are directly involved in initiation of the canonical UPR: ATF6, PERK, and IRE1. ATF6 is released and activated by dissociated *Bip* from the luminal side of ER. High dosage of LCBs caused a significant decrease in ATF6 mRNA expression in HFFD-fed mice ( $p < 0.01$ ; Figure 4B). Figure 4C



**Figure 3** – Effects of long-chain bases on c-Jun NH<sub>2</sub>-terminal kinase and inhibitor  $\kappa$  kinase  $\beta$ /nuclear factor (NF)  $\kappa$ B pathways in epididymal adipose tissue of obesity mice. (A) p-c-Jun NH<sub>2</sub>-terminal kinase 1 protein expression; (B) p-inhibitor  $\kappa$  kinase  $\beta$  protein expression; (C) nuclear NF $\kappa$ B protein expression; (D) cytoplasmic NF $\kappa$ B protein expression. Data are expressed as mean  $\pm$  standard deviation ( $n = 12$ /group). Multiple comparisons were done using one way ANOVA analysis followed by Tukey's test. <sup>##</sup>  $p < 0.01$  versus control; <sup>\*</sup>  $p < 0.05$ , <sup>\*\*</sup>  $p < 0.01$  versus high-fat fructose diet (HFFD).

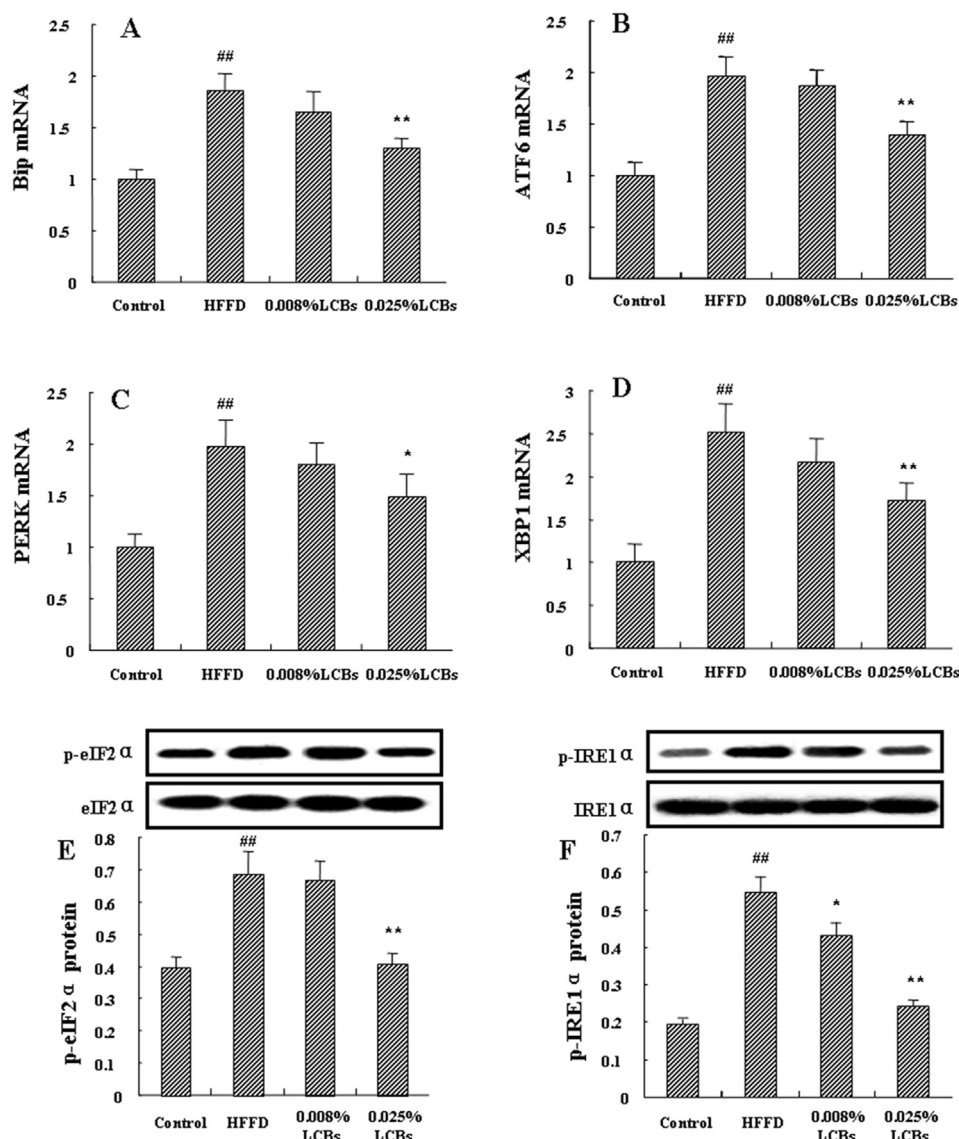
showed that the mRNA expression of PERK was also reduced by LCBs ( $p < 0.05$ ). Autophosphorylated PERK can activate eIF2 $\alpha$ , the target gene to attenuate the rate of translation initiation. Treatment with high dosage of LCBs significantly promoted phosphorylation of eIF2 $\alpha$  ( $p < 0.01$ ), and this tendency was also observed in low dosage of LCBs-treated mice ( $p < 0.05$ ; Figure 4E). IRE1 $\alpha$  is activated through autophosphorylation and specifically splices XBP1 mRNA, which induces UPR target genes. As shown in Figure 4D and F, in the LCBs-fed mice, phosphorylated IRE1 $\alpha$  and XBP1 mRNA expression were significantly reduced ( $p < 0.01$ ). These results suggest that LCBs can alleviate ER stress through inhibition of Bip-triggered UPR.

#### 4. Discussion

Sustained inflammation and ER stress can result in hyperglycemia [11,26]. In the previous studies, LCBs from *C. frondosa* could reduce blood glucose levels in obesity mice [21,22]. Here, the effects of LCBs on alleviation of hepatic ER stress and

systemic inflammation were further investigated. The results showed that LCBs reduced body weight and the levels of stressors of ER stress and inflammation, ROS and FFAs. LCBs also mitigated ER stress and inflammatory response in the liver of obesity mice.

We showed that LCBs significantly lowered body weight gain and epididymal adipose weight, suggesting an anti-obesity effects of LCBs. High-calorie diet feeding-induced obesity causes obvious inflammatory response [27]. Adipose tissues are familiarized as the production and secretion of cytokines, which are interrelated with inflammation [28]. Our results showed that LCBs inhibited proinflammatory mRNA expressions and increased anti-inflammatory mRNA expression in epididymal adipose tissue of obesity mice, suggesting that LCBs-reduced epididymal adipose weight may cause the decreases in TNF- $\alpha$ , CRP, MIP-1, IL-1 $\beta$ , and IL-6, and the increase in IL-10. Circulated cytokines concentrations in HFFD-fed mice were also regulated toward to the levels in control group when supplemented with LCBs, which indicates that LCBs mitigate inflammatory response through regulation of inflammatory cytokines.



**Figure 4 – Effects of long-chain bases on ER stress in the liver of obesity mice. (A) Bip mRNA expression; (B) ATF6 mRNA expression; (C) phosphoenolpyruvate kinase mRNA expression; (D) XBP1 mRNA expression; (E) p-eIF2 $\alpha$  protein expression; (F) p-IRE1 $\alpha$  protein expression. Data are expressed as mean  $\pm$  standard deviation ( $n = 12$ /group). Multiple comparisons were done using one-way ANOVA analysis followed by Tukey's test. <sup>##</sup>  $p < 0.01$  versus control; <sup>\*</sup>  $p < 0.05$ , <sup>\*\*</sup>  $p < 0.01$  versus high-fat fructose diet (HFFD).**

Inflammatory response occurs in part through I $\kappa$ B/ $\text{NF-}\kappa\text{B}$  pathway [29]. Abnormal stimulation by ROS, FFAs, or proinflammatory cytokines induces the inhibitor of  $\text{NF-}\kappa\text{B}$  phosphorylation and subsequently promotes  $\text{NF-}\kappa\text{B}$  nuclear translocation [30]. The nuclear  $\text{NF-}\kappa\text{B}$  then regulates the transcription of numerous inflammatory genes, such as TNF- $\alpha$ , MIP-1, and IL-6 [29,31]. In this study, LCBs significantly inhibited the activation of I $\kappa$ B and suppressed the nuclear translocation of  $\text{NF-}\kappa\text{B}$ , suggesting that LCBs can alleviate inflammatory response through inhibition of the I $\kappa$ B/ $\text{NF-}\kappa\text{B}$  pathway. JNK signaling is another pathway of progression of chronic low-grade systemic inflammation. Phosphorylated JNK can result in inflammation and its complication [32]. Our results showed that phosphorylated JNK1 was decreased by LCBs supplementary in obesity mice. This indicates that LCBs-

deactivated JNK signal also exerts an important effect on the alleviation of inflammatory response.

Studies have proposed ER stress to be involved in the pathophysiology of inflammation [7,33]. Anabolic ER chaperones Bip mRNA expression, and the amplified mRNA levels of ATF6, phosphoenolpyruvate kinase, and XBP1 and phosphorylation status of eIF2 $\alpha$  and IRE1 $\alpha$  suggest obvious ER stress in the liver of high-calorie diet-fed mice. When treated with LCBs from *C. frondosa*, the obesity mice exhibited hypoactive ER stress, showing the significant decreases in the mRNA and phosphorylated protein expressions of the aforementioned UPR-related genes. The inhibition of ER stress was coupled with an improvement in inflammatory response by LCBs. Kim et al [34] reported that when interrupted ER stress response, TNF- $\alpha$  and IL-1 $\beta$  mRNA

expressions were reduced through the adynamic JNK and NF $\kappa$ B pathways in hepG2 cells. Pan et al [35] showed that the lower response of ER stress resulted in lower mRNA and protein expression of TNF- $\alpha$  and IL-1 $\beta$  in the liver of high-fat diet-fed rats. In the present study, LCBs-reduced ER stress response also caused decreases in proinflammatory cytokines levels and increase in IL-10 level. This indicates that LCBs can improve inflammatory response through intervention of ER stress.

In the earlier stages of ER stress conditions, increased level of mitochondrial calcium alters metabolism and eventually ROS production [10]. The elevation of ROS in the mitochondria induces ER to release calcium continuously through a channel in the membrane of ER, sending a feedback signal [36]. The increased ROS triggers inflammatory response through several complex mechanisms, including phosphorylation of JNK and NF $\kappa$ B nuclear translocation [37,38]. Li et al [4] confirmed that alleviation of ROS-associated ER stress resulted in a reduction in IL-1 $\beta$  secretion. Bhuvanewari et al [31] also showed that impaired ER stress response can lead to the reduction in intracellular ROS production, and subsequently alleviate inflammation through JNK and NF $\kappa$ B pathways in HFFD-fed mice. Our results showed that LCBs reduced circulating ROS and hepatic ROS production associated with alleviation on ER stress in obesity mice. Meanwhile, the JNK and NF $\kappa$ B pathways were also blocked by LCBs, suggesting that suppression of ROS production by LCBs can counterbalance the deleterious outcome of ER stress and its response to inflammation.

FFAs typically exacerbate ER stress-triggered inflammation [6]. Tampakakis et al [39] found that both peripheral blood mononuclear cells and vascular endothelial cells showed severe ER stress, when suffered with FFAs. FFAs can also activate I $\kappa$ K $\beta$  pathways and finally lead to inflammation in adipocytes [40]. Neacsu et al [41] showed that TNF- $\alpha$  and activated JNK proteins were downregulated when the FFAs levels decreased. Moreover, overloaded FFAs and proinflammatory cytokines can increase ER stress-induced mitochondrial ROS product, which finally activates NF $\kappa$ B and JNK pathways [42]. In the present study, high-calorie diet-fed mice exhibited high levels of ROS in serum and liver, and interestingly, the elevations were decreased by LCBs supplementary. These observations indicate that LCBs mitigate ER stress and inflammation through negative induction of FFAs and ROS.

## 5. Conclusions

This study showed that LCBs were capable of alleviation of ER stress and inflammatory response in high-calorie feeding-induced obesity mice. Downregulated Bip mRNA and the blocking of three branches of UPR (IRE1 $\alpha$ /XBP1, PERK/eIF2 $\alpha$ , and ATF6 signaling) explained the underlying mechanism of LCBs-mitigated ER stress. Inactivation of JNK and I $\kappa$ K- $\beta$ /NF $\kappa$ B pathways caused the attenuation of inflammatory response by LCBs through regulating inflammatory cytokine production. To the best of our knowledge, this was the first studying in the existing literature on this topic and it provided important information on the utilization of LCBs from sea cucumber against inflammation.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jfda.2016.10.011>.

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